

Enzymes in the biosynthesis of aliphatic
tricarboxylic acids in *Penicillium spiculisporum*

by

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1. SUMMARY

The biosynthesis of spiculisporic acid has been elucidated and the enzyme responsible for its formation has been studied. Decylcitric acid, which is a new compound, has been detected in a strain of *Penicillium spiculisporum*, and its formation has been established by enzymatic methods. The properties of the enzymes have been studied particularly with respect to their dependence on fatty acyl-CoA concentrations. The substrate binding to the enzymes suggests a compulsory-order ternary complex mechanism, where the keto acid binds first. Citrate synthase (EC 4.1.3.7.), which catalyses an analogous reaction, has also been isolated from the organism, and its properties have been compared particularly with those of decylcitrate synthase. Medium length fatty acid synthetase and ATP citrate lyase (EC 4.1.3.8.) have also been purified from *P. spiculisporum*. The properties of the primary metabolic enzymes are similar to those from mammalian sources, and the lipid synthesis may well proceed through mitochondrial citrate leakage, as suggested for mammalian lipid synthesizing tissues. No strong regulatory mechanism has been found for the enzymes investigated.

The thesis is based on the following papers:

- I. The enzymic synthesis of spiculisporic acid. Gatenbeck, S. & Måhlén, A. (1966) Proc. Congr. Antibiotics in Prague 1964, p. 540-541, Butterworth, London.
- II. A metabolic variation in *Penicillium spiculisporum* I. Production of (+)- and (-)-decylcitric acids. Gatenbeck, S. & Måhlén, A. (1968) Acta Chem. Scand. 22, 2613-2616.
- III. A metabolic variation in *Penicillium spiculisporum* II. Purification and some properties of the enzyme synthesizing (-)-decylcitric acid. Gatenbeck, S. & Måhlén, A. Acta Chem. Scand. 22, 2617-2623.
- IV. Properties of 2-decylcitrate synthase from *Penicillium spiculisporum*, Måhlén, A. (1971) Eur. J. Biochem. 22, 104-114.
- V. Purification and some properties of citrate synthase from *Penicillium spiculisporum*, Måhlén, A. (1972) Eur. J. Biochem. 29, 60-66.
- VI. Purification and some properties of ATP citrate lyase from *Penicillium spiculisporum*. Måhlén, A. (1973) Eur. J. Biochem in press.
- VII. Purification and some properties of 2-decylhomocitrate synthase from *Penicillium spiculisporum*. Måhlén, A. (1973) Eur. J. Biochem. in press.

2. INTRODUCTION

The production in moulds and other microorganisms of so-called secondary metabolites, i.e. those which are not directly involved in the life maintaining primary metabolism, is of considerable interest to man. Among the substances are found many with clinically valuable antibiotic properties or other industrial use. The substances are often species-specific and show a tremendous variation in the synthetic capabilities of the organisms. The study of the enzymes involved in these reactions, provides increased knowledge of the principles of biological catalysis, and may give information, which is otherwise inaccessible, and applicable to primary metabolic enzymes. The future use of purified secondary biosynthetic enzymes bound to matrices, will make it possible for the industrial production of valuable natural products, which are otherwise difficult to synthesize chemically in clean reactions.



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3. ENZYME STUDIES OF SECONDARY BIOSYNTHETIC PATHWAYS IN MOULDS

The majority of the secondary products in moulds are aromatic acetate-malonate condensation products, named polyketides after the hypothetical intermediate. They occur in numerous variations with changes in ring size, number of rings and degree of substitution and other reactions. The polyketides are also found in lichens. Their biosynthesis has been known for a rather long time (1), but only relatively recently have enzymes been purified that are capable of performing the aromatisation reaction. Thus, in 1961, Lynen and Tada reported the cellfree synthesis of 6-methyl-salicylic acid (2). Later Dimroth, Walter and Lynen have purified the enzyme from *Penicillium patulum* and studied its properties (3). Gatenbeck, Hermodsson and Sjöland have partially purified alter-nariol synthetase from *Alternaria tenuis*, an enzyme making and folding a linear polymer of one acetyl-CoA and six malonyl-CoA (4,5). Orsellinic acid synthetase, which has been isolated by Gaucher and Shepherd from *Penicillium madriti*, is another example of an enzyme catalysing an acetate-malonate condensation (6). One characteristic of these enzymes is that they are large, probably of the same type as fatty acid synthetase. They are in fact difficult to separate from fatty acid synthetase in the purification procedure.

Other enzymes related to secondary metabolism, and involved in reactions following the primary synthesis have been studied, such as decarboxylases, methyl

transferases, dehydrogenases etc.

Thus, relatively few enzymes responsible for the primary events in natural product biosynthesis have been isolated to date. One reason for this may be their relatively poor stability in cell-free solution, but as soon as means to overcome this problem are found interesting studies in this special enzymology will be possible.

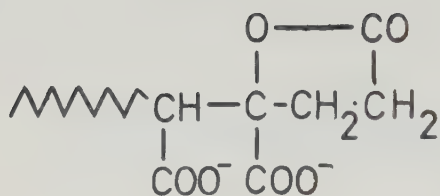
4. LONG CHAIN ALIPHATIC DI- AND TRICARBOXYLIC ACIDS

a. Occurrence.

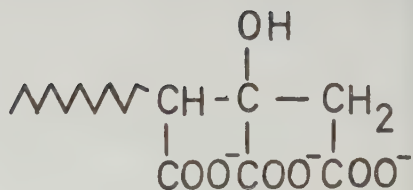
The long chain di- and tricarboxylic acids are essentially microbial in occurrence, although one of them, pedicellic acid, has been found in a plant and A.L. Lehninger (personal communication) has suspected their existence in mammalian mitochondria. Most of the structures are found in lichens, but some are of fungal origin. Since the lichens are symbiotic organisms, the acids may be entirely derived from fungal cells. In species capable of producing them, the acids often occur in large quantities. A short chain acid of the same type is 2-methylcitrate, which has been reported as an anomalous metabolite in man (7).

b. Structures.

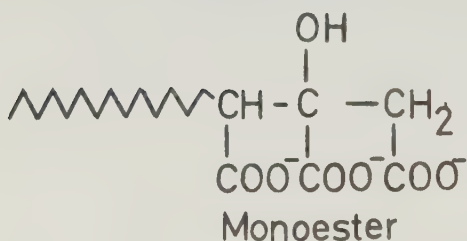
Examples of structures of the aliphatic acids are found in Fig. 1. The acids are typically of the structural type 2-hydroxy-alkane-1,2,3-tricarboxylic acid, but spiculisporic acid is a 3-hydroxy-alkane-1,3,4-tricarboxylic acid. Lactonisation is a common feature. Another common type is the alkane (or alkene)-2,3-dicarboxylic acid. More complex structures derived from two acids of this type are exemplified by glauconic acid and Rubratoxin B. All the substances are optically active and contain two asymmetric carbons, usually C-2 and C-3. The absolute configurations of rangiformic and roccellic acid have been determined by Åkermark by stereospecific synthesis (23). Decylcitric acid has been isolated in two forms with the same formula but with different optical



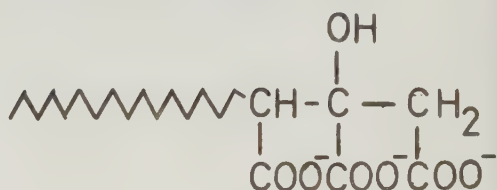
Spiculisporic (8)
C₁₂ + C₅



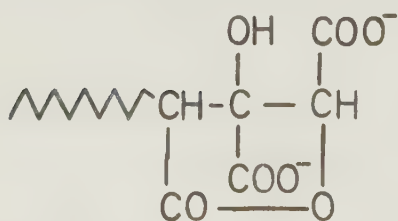
2-Decylcitric (II)
C₁₂ + C₄



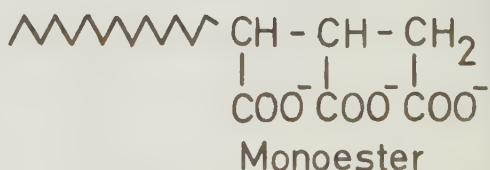
Caperatic (9)
C₁₆ + C₄



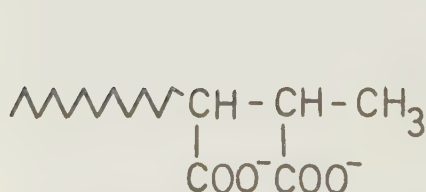
Agaricic (10)
C₁₈ + C₄



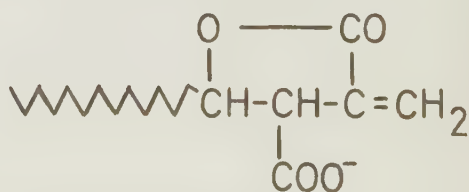
Minioluteic (11)
C₁₂ + C₄



Rangiformic (12)
C₁₆ + C₄

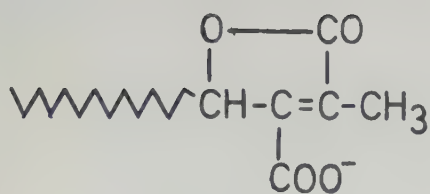


Roccellic (13)
C₁₄ + C₄



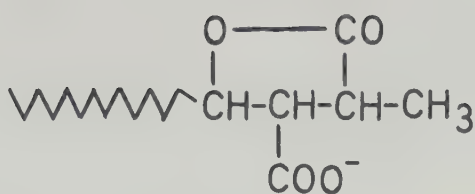
Protolichesterinic (14)
C₁₆ + C₄

Fig. 1.



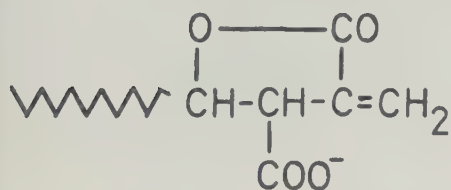
Lichesterinic (15)

$\text{C}_{16} + \text{C}_4$



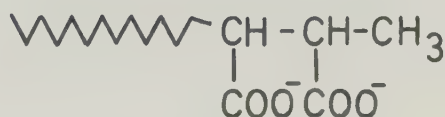
Nephromopsic (16)

$\text{C}_{16} + \text{C}_4$



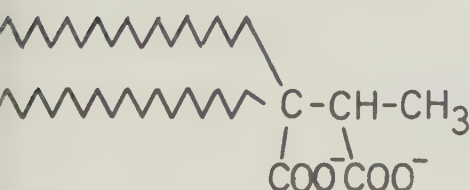
Nephrosterinic (17)

$\text{C}_{14} + \text{C}_4$



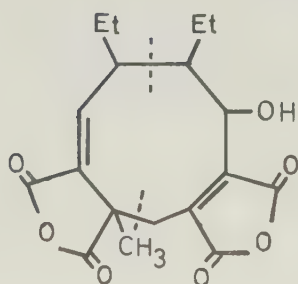
Pedicellic (18)

$\text{C}_{15} + \text{C}_4$



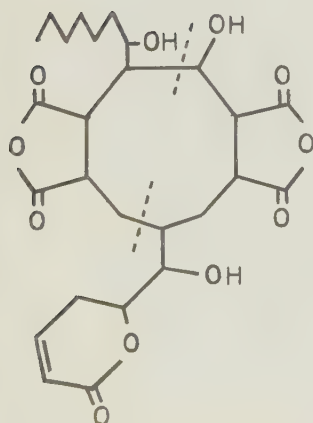
Fomentaric (19)

?



Glauconic (20)

$2(\text{C}_6 + \text{C}_4)$



Rubratoxin B (21, 22)

$2(\text{C}_{10} + \text{C}_4)$

Fig. 1.

rotation (II). The length of the aliphatic chain is between C_{10} and C_{18} and there is one odd-numbered C_{13} chain in pedicellic acid. The cyclised compounds are derived from acids with side chains C_4 in glauconic acid and C_8 in Rubratoxin B.

c. Properties.

The aliphatic di- and tricarboxylic acids are highly polar substances, and are of potential economic interest as detergents. Substances like decylcitric acid may be especially useful because of high water solubility and surface activity even at rather low pH, where few good detergents are available. The pK_a values for the three carboxyls fall between 3 and 6 (unpublished), like those of citric acid. The pK_a values of spiculisporic acid are between 4 and 5, and this substance, as may be deduced from its structure, is less water soluble. The substances are readily soluble in ether, but almost insoluble in petroleum-ether (II). Decylcitric and spiculisporic acid form poorly soluble salts with Ba^{2+} , Ca^{2+} , Mg^{2+} and Cu^{2+} (Cfr 8). The water solutions of the substances form a stable foam, which presents technical problems in the production of the acids by moulds. It has not been possible to use aerated tank culture; instead a large number of Erlenmeyer flasks have been used in shake culture. Decylcitric acid has been found in yields of about 6 g/l in the culture medium, whereas with spiculisporic acid yields of 2 g/l have been recorded. The optical forms of decylcitric acid are difficult to resolve on a preparative scale, but analytically they are nicely separated on TLC (II). The decylcitric acids give, in contrast to spiculisporic

acid, a red compound with pyridine and acetic anhydride, as described for citric acid (24). The biological properties of the acids from *P. spiculisporum* are unknown, but a concentrated solution, such as found in a culture medium, should have a profound influence on membrane systems and it is astonishing that the mould can survive its environment. One of the isomers of decylcitric acid can be re-utilised by the mould (II): thus the acid may be regarded as an extracellular energy-storage product. The acids are strong protein denaturants and can be used for detergent electrophoresis instead of dodecyl sulphate. Antibacterial properties of the long chain acids have been reported (25).

d. Biosynthesis.

The biosynthesis of the two acids from *P. spiculisporum* has been clarified by isotope incorporation studies and isolation of the enzymes involved in the synthesis (I, II, III, IV, VII). The reaction is, as proposed by several authors from inspection of the structures of the substances, a condensation of fatty acyl-CoA and an α -keto acid, either oxalacetate or α -ketoglutarate, in analogy with the formation of citrate by citrate synthase. The same mechanism of synthesis can easily be applied to agaricic and cape-ratic acid and possibly also to minioluteic acid, with hydroxylacetate as the condensation partner.

The citrate synthase type reaction necessitates that the carbonyl group of the keto acid appears as a hydroxyl in the product. However, most of the structures in Fig. 1 lack this hydroxyl and must have been subject to secondary reactions after the condensation.

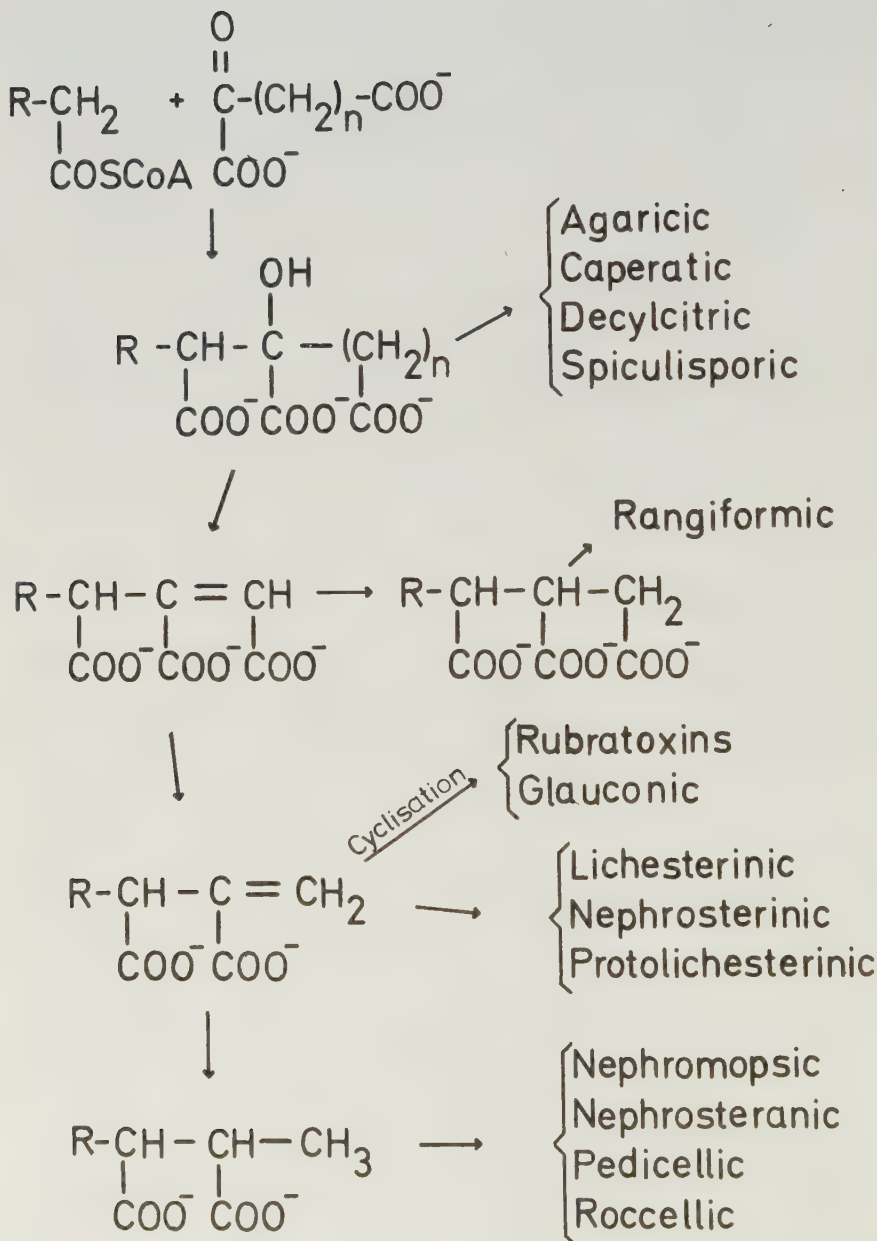


Fig. 2. Suggested biosynthesis of the acids.

Most of the structures could formally be derived from pyruvate and a fatty acid, but, interestingly, not a single one has retained the hydroxyl group formed in the condensation. The biosynthesis of two of the acids possibly derived from pyruvate, glauconic acid from *Penicillium purpurogenum* and protolichesterinic acid from *Cetraria islandica* has been investigated by Bloomer et al. (26-30), and he arrives at the conclusion that the C_3 fragment is derived from a C_4 acid. For the enzymes performing this type of condensation - citrate synthase, decylcitrate synthase and decylhomocitrate synthase - it is known that both the carboxyls are needed for the reaction (31,32 IV,VII). The scheme in Fig. 2 can therefore be suggested for the synthesis of all the acids. The dehydration first suggested, aids in the subsequent decarboxylation, and the unsaturated dicarboxylic acid then formed can be hydrogenated to give the acids with an apparent three carbon moiety. The cyclisation reaction leading to glauconic acid has been indicated from isotope experiments by Bloomer et al. (26).

The synthesis of the secondary metabolites is limited to a rather short period in the growth of a mould culture. One might expect that non-nitrogen containing compounds at least, should be synthesized in the stationary phase, when the nitrogen source is exhausted, and only glucose is left in the medium. The synthesis is actually maximal in the growth phase, where there should be no shortage of any growth factors. This has been observed with 6-methylsalicylate synthetase (3), orsellinate synthetase (6), anthraquinone production (33) and, interestingly, also in the lipid and steroid production of *Rhizopus* (34).

There may be either a sensitive mechanism which switches on secondary production at a defined residual nitrate concentration in the medium or the ratio between glucose and nitrate may be the crucial factor. Similar drastic metabolic changes occur in rat liver after starving and refeeding and in mammary gland at the onset of lactation. A common regulatory mechanism for these events can not be excluded.

The reactions of the primary metabolism and those of the secondary metabolism are very often similar, and the opinion may be advanced that the enzymes are the same, but in different environments. This is supported, for instance, by the observation that fatty acid synthetase can, under special circumstances synthesize triacetic lactone, which is a polyketide of the simplest kind (35,36). The evidence from this and other organisms, however, discloses that the enzymes of primary and secondary metabolism are entirely different proteins.

5. BIOSYNTHESIS OF THE ACIDS FROM *PENICILLIUM* *SPICULISPORUM*

a. Spiculisporic acid.

Several authors have postulated that spiculisporic acid should arise from the condensation of laurate and α -ketoglutarate. This has been confirmed by labelling the acid from radioactive glutamate (I). A remarkable aspect of this experiment was that as much as 15% of the radioactivity incorporated was found in the fatty acid part of the molecule. This suggests that there are pathways for conversion of ketoglutarate into acetyl-CoA or malonyl-CoA in the mould. The condensation, which requires laurate to be esterified with CoA, is of a well known type; the most prominent example of such a reaction is that of citrate synthase, where acetate in the form of acetyl-CoA condenses with oxalacetate. Other examples of enzymes performing this type of reaction are malate synthase and isopropylmalate synthase (37). The biosynthesis of spiculisporic acid also requires lactonisation of the 2-decylhomocitric acid, which is likely to be the primary product of the reaction. The lactonisation may be catalysed enzymatically, but it occurs spontaneously as well (8).

b. 2-Decylcitric acid.

Decylcitric acid was found in a variant strain of *Penicillium spiculisporum*, which arose spontaneously in this laboratory (II). Since the acid was enzymatically prepared before it was isolated from the

culture medium, no isotope incorporation experiments in whole cells were necessary. The first substrate, which was found to be active, was aspartate, which gave rise to an ether- but not petroleumether soluble radioactive substance, when incubated together with lauroyl-CoA-1-¹⁴C in a crude enzyme extract. Later, oxalacetate was found to be an even better substrate, and aspartate obviously was transaminated by an appropriate enzyme with available keto acids in the crude cell extract. The culture medium was then found to contain two acids with the same formula, but with different optical rotation; the (-) form being identical with the one found in an enzymatic preparation, and the (+) form being prevalent in the medium.

An enzyme capable of interconverting the optical forms was found in a crude enzyme extract, but during the experimental conditions, the equilibrium was in the direction of (-)-decylcitric acid. It is not known where the difference between the isomers is to be consigned; either C-2 or C-3 are possible.

X-ray crystallographic determination has been suggested (J.P. Glusker personal communication), but unfortunately the decylcitric acids are very difficult to crystallise. The absolute configuration determinations of aliphatic lichen acids made by Åkermark (23) only concern acids lacking the hydroxyl group formed in the condensation. Since dehydration and hydrogenation may involve changes in configuration, his data unfortunately do not reveal anything concerning the stereochemical relationships in the condensation reaction. It would be interesting to compare the stereochemistry of the *P. spiculisporum* synthases with that of citrate synthase (38-40).

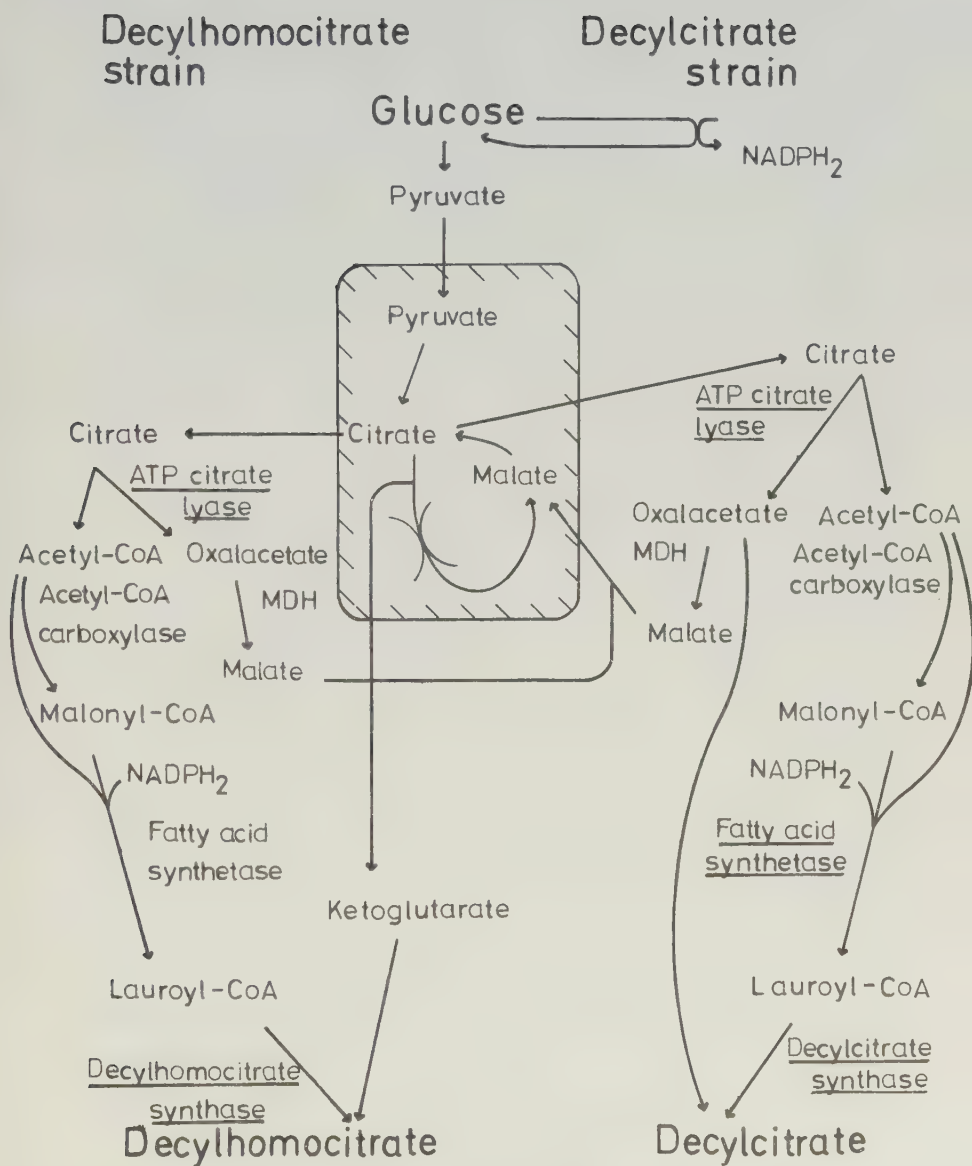


Fig. 3. Hypothetic pathways to the *P. spiculisporum* acids.

c. Metabolic pathway and regulation.

A possible pathway leading to the acids in *Penicillium spiculisporum* is given in Fig. 3. Enzymes which have actually been isolated from the two strains are underlined. In the decylcitrate strain the biosynthesis fits very well with the theory of mitochondrial citrate export (41), which has been proposed for rat liver and other animal tissues. The crucial enzyme in this pathway is ATP citrate lyase, the activity of which in mammalian tissues parallels the rate of fatty acid synthesis or other acetyl group requiring processes. ATP citrate lyase has been found in large amounts in both the strains.

One striking feature in the biosynthesis of the acids is the high rate of fatty acid synthesis. Secondary products of acetate origin in moulds are generally nitrogen free condensation products with about the same oxidation level as acetate. This could reflect the usual growth conditions, where the nitrogen source is limiting and the glucose is in large excess, and the production of secondary metabolites could be considered as a simple means of removing excess carbon metabolites in the cells. The *P. spiculisporum* acids are unusual as "over-flow" metabolites in that they require a large supply of reducing equivalents (NADPH), and the synthesis is, in contrast to the formation of aromatic acetate-malonate substances, energetically expensive. The source of these amounts of NADPH₂ is unknown, but an active pentose phosphate cycle offers one possible explanation (42).

The mechanism of formation of malonyl-CoA is also unknown; either acetyl-CoA is carboxylated by acetyl-

-CoA carboxylase, which is the usual pathway (43), or oxalacetate is oxidatively decarboxylated. This last mechanism is known in plants (44), and has also been reported from a *Penicillium* species (45).

Lauroyl-CoA which is the main product of fatty acid synthetase in *P. spiculisporum* is needed in large amounts, but the steady state concentration can be quite low, since both decylcitrate synthase and decylhomocitrate synthase have very low K_m values for lauroyl-CoA. The compartmentalisation of fatty acyl-CoA in the cytoplasm poses interesting problems. All such derivatives have high affinity for proteins, and the concentration of free fatty acyl-CoA in the cytoplasm should accordingly be very low. A recent paper by Sumper and Träuble (42) suggests that fatty acyl-CoA, which has been synthesized is transported via two-dimensional surface diffusion in lipid membranes to the site of consumption. Such a transport system would increase the efficiency of the synthesis of fatty acid derivatives, reduce the general detergent inhibition on almost all enzyme systems characteristic of fatty acyl-CoA and lower the product inhibition on fatty acid synthetase. There is no direct evidence of a lipid transport system in this or any other system, but the in vitro experiments are suggestive.

A strain difference is evident in the keto acids. The biosynthesis in the decylcitrate strain fits nicely with the citrate export theory, where the oxalacetate formed by ATP citrate lyase can be partially used by decylcitrate synthase and partially reduced to malate and re-enter the mitochondria in exchange for more citrate (47). A mechanism of this type would allow for an efficient transfer from the mitochondria

to the cytoplasm of acetyl groups, which are needed in six-fold excess over oxalacetate in the biosynthesis. In the spiculisporate strain the origin of the ketoglutarate for the biosynthesis is less easily explained. Essentially, two ways are possible. Either ketoglutarate is formed from extramitochondrial citrate with cytoplasmic aconitase and isocitrate dehydrogenase (48), or it is transported directly from the mitochondria, for instance in exchange for malate (47). In this strain oxalacetate formed by ATP citrate lyase is, after reduction, available for the citrate- malate exchange and citrate- ketoglutarate exchange.

One more problem remains in the biosynthesis. Since the final products are strong protein denaturants, they have to be compartmentalised and efficiently transported out of the cells. An interface diffusion mechanism as proposed for fatty acyl-CoA would be a solution to this problem.

Biochemically the best known material is rat liver, to which studies on the difficult problems concerning metabolic flow and regulation have been directed. One point of control of the lipid synthesis in this tissue is acetyl-CoA carboxylase which is activated by citrate (49). It would then seem that the amount of cytoplasmic citrate would regulate the lipid synthesis provided that ATP and NADPH are not limiting. One has then turned the interest towards citrate synthase in search for a basic lipid regulatory mechanism. ATP, which here is an inhibitor competitive with acetyl-CoA, has been suggested as a regulator by Atkinson (50). This is however a rela-

tively poor regulator and the inhibition is partially reversed by Mg^{2+} . Others have stressed the importance of the oxalacetate concentration, which seems to be limiting for the rate of citrate synthesis (50a). In the case of a large citrate export from the mitochondria, oxalacetate, of course, should be even more important, since it is not regenerated by the citric acid cycle. The enzyme which then is important is pyruvate carboxylase, which is an allosteric enzyme and accordingly subject to powerful regulation (51). An effector is acetyl-CoA, which favours the reaction, thereby reducing the imbalance between oxalacetate and acetyl-CoA, such as arising from high pyruvate influx and low citric acid cycle activity. The membrane transport mechanisms might be considered as possible regulators, however, the data so far presented indicate simple Michaelis-Menten behaviour, i.e. the concentrations of the substrates alone determine the rate of transport.

In *Penicillium islandicum*, Gatenbeck and Sjöland have studied the metabolic events at the onset of production of anthraquinones (33), which are acetogenins like the acids dealt with in this work, although their formation does not involve fatty acid synthesis. They observed a lowered citric acid cycle activity and a rise in the cellular citrate concentration at the beginning of the secondary production. The most prominent environmental factor was the near exhaustion of the nitrogen source in the medium, and it seems reasonable that the capacities of the mould then should be diverted to the synthesis of carbon substances. The observations in *P. islandicum* lend support to the importance of citrate as donor of

acetyl groups for acetogenin biosynthesis.

In *P. spiculisorum* no whole cell metabolic measurements have been made, but instead the enzymes involved in the hypothetical pathway have been isolated and studied. The specific activities of citrate synthase, ATP citrate lyase, fatty acid synthetase and decylcitrate synthase are of the same magnitude in the crude enzyme extract, based on acetyl group turn-over, but they are rather low in comparison with the in vivo rate of synthesis (VI). Usually isolated enzyme activities are well above the in vivo rate of a pathway, however the measured activities are subject to error, as discussed in VI and VII, and the enzymes studied may account for the rate of decylcitrate synthesis. Such an investigation does not exclude of course, the participation of other pathways parallel with the one suggested.

Vanek et al. (52) have investigated the properties of citrate synthase in *Streptomyces aureofaciens* with particular reference to possible regulatory effects on the chlorotetracycline biosynthesis. They arrived at the conclusion that citrate synthase is inhibited by ATP and that this makes acetyl-CoA available for acetogenin biosynthesis. Their enzyme is like the *P. spiculisorum* enzyme actually rather insensitive to inhibition by ATP, if compared with citrate synthases from other sources. The in vivo inhibition will, however, depend on the concentrations of substrates and inhibitors in the actual compartment, and in vitro kinetic measurements can only provide a hint about what is possible. A comparison between the actinomycete and this mould is further complicated by their different subcellular organisation.

P.A. Srere (personal communication) has investigated species of bacteria and yeast without success in search for ATP citrate lyase, which in mammalian tissue is a good indicator of cytoplasmic acetyl-CoA utilisation.

In conclusion, it can be said that the evidence in *Penicillium spiculisporum* is compatible with a mitochondrial citrate export mechanism, and that no strong regulatory mechanism for the secondary biosynthetic pathway has been found in this investigation.

6. ENZYMES IN THE POSTULATED PATHWAY

a. Common features.

A feature common to all the enzymes dealt with in this work is that they are dependent on CoA. Such enzymes are found in various enzyme classes, but the types of reaction essentially involve either attack on the α -carbon or on the carbonyl group of an acyl-CoA compound. Reactions involving the carbonyl group may include formation of an acyl-enzyme as in fatty acid synthetase (53), and then there is no absolute requirement of the whole CoA molecule. It can be replaced by pantetheine or even shorter thiols (54). Reactions involving the α -carbon of an acyl-CoA, on the other hand, require the whole CoA molecule for activity, as exemplified by citrate synthase (55).

Attempts to prepare CoA analogues suitable for affinity chromatography of these enzymes have included the synthesis of 1,N⁶-etheno-CoA and ω -substituted fatty acyl-CoA. 1,N⁶-etheno-CoA, synthesised using the method employed on adenosine by Kochetov et al. (56), did not show coenzymic activity, either in the ATP citrate lyase reaction (VI) or, as the acetyl derivative, in the citrate synthase reaction (unpublished). This can be interpreted as indicating that the N⁶ amino group is required in the binding of the substrate, but on the other hand, the introduction of the extra ring to the adenine structure will cause a change in the size of the molecule, which may be of importance. ξ -ATP, ζ -ADP and η -AMP, which are similar derivatives, are all enzymatically active (57), and it can not be excluded that the CoA mole-

cule, with its long side chain, has a specific folding at the active site, which is made impossible by the substitution.

The preparation of ω -substituted fatty acyl-CoA derivatives has given substrate analogues, which are partially active (IV) but the attachment of these to a gel have failed so far. The acyl-CoA molecule contains many reactive groups, but the thioester linkage is especially sensitive, and this may offer one explanation for the difficulties encountered.

b. Citrate synthase.

Citrate oxaloacetate lyase (CoA acetylating)

EC 4.1.3.7.

Citrate synthase is a relatively well known enzyme and has been isolated from a variety of organisms. Most data have been collected from the pig heart enzyme isolated in 1951 by Stern and Ochoa (58) and now commercially available, but there are also reports on other animal and microbial enzymes and a few plant enzymes. In eucaryotes, citrate synthase is considered to be exclusively located in the mitochondrial matrix (48), with the exception that isoenzymes have been found in plant glyoxisomes and mitochondria (59). One major grouping divides them into re- and si-citrate synthase giving different stereochemistry in the enzymic treatment of the citrate formed (60). Whereas si-citrate synthase, which is the eucaryotic type, produces a citrate, which is attacked from the oxalacetate end in subsequent steps in the cycle, re-synthases which occur in a few strict anaerobes, give a citrate, which is attacked from the acetate end (61). Some bacterial enzymes are under allosteric

control, such as the *E. coli* enzyme (62), and possible control by NADH_2 , AMP, ATP and ketoglutarate concentrations has been claimed (62-66). The eucaryotic enzymes are more similar in their properties.

Typically, the eucaryotic citrate synthase is a dimer with a molecular weight of 70 000 - 100 000 (67). K_m for the substrates is 5 - 50 μM and it is independent of free SH groups and metal ions (68). Exceptional properties are found, for instance in the mango fruit enzyme, which is sensitive to SH reagents in the absence of substrates (69), trout tissue, which contains isoenzymes with temperature dependent K_m (70) and Phaseolus enzyme for which indoleacetate dependence has been claimed (71,72). ATP is generally an inhibitor competitive with acetyl-CoA, but this inhibition is reduced by Mg^{2+} . The discussion of the physiological role of the ATP inhibition is thus complicated by the difficulty in estimation of the concentrations of several reactants. Succinyl-CoA is also an inhibitor, which may be of physiological significance (73).

Citrate synthase from *Penicillium spiculisporum* (IV) has properties similar to those of the pig heart enzyme (68) so far as investigated. It is however slightly smaller, and the ATP inhibition is less pronounced. It has been purified to a reasonable degree, but its tendency towards aggregation has discouraged attempts to achieve a homogeneous protein.

c. ATP citrate lyase.

ATP: citrate oxaloacetate-lyase (CoA acetylating and ATP dephosphorylating) EC 4.1.3.8.

ATP citrate lyase is less well studied, although discovered by Srere and Lipmann as early as 1953 (74). It is a large and unstable enzyme, and is not commercially available. It is particularly found in those tissues, which have a high rate of fatty acid synthesis or other types of cytoplasmic acetyl-CoA utilisation. Very little is known about its occurrence in organisms except animals; only one report from mango fruit and one from *Bacterium succinicum* are at hand, but the enzymes were not purified (75,76). The citrate cleavage enzyme, e.g. from rat liver, is cytoplasmic and occupies a key role in the transfer and utilisation of mitochondrial acetyl-groups in the cytoplasm.

The rat liver enzyme is SH dependent and as usual Mg^{2+} or Mn^{2+} is required for complexing with ATP, which is a cosubstrate (77,78).

The properties of the *P. spiculisporum* enzyme are similar (VI). The molecular size has not been determined, but is of the same magnitude. The apparent K_m values give the same impression i.e., the enzyme is saturated by low concentrations of CoA and the saturating levels of citrate and MgATP are relatively high. The rat liver enzyme forms a phospho enzyme which seems to be an intermediate in the reaction (79), but for this mould the data on this aspect are contradictory, and further investigations are necessary.

Penicillium spiculisporum thus is a very good

source of an ATP citrate lyase, which parallels the rat liver enzyme, and it may well fulfil the same role as postulated for the animal enzymes.

d. Fatty acid synthetase.

The fatty acid synthesis in *Penicillium spiculisporum* offers an interesting problem, but as yet relatively little work has been done on it, and the data given here are unpublished.

When a lipid extract is prepared from the mycelium in the usual way (80) (which excludes the alkyltricarboxylic acids), and the fatty acids determined by GLC, a normal spectrum is found with predominating linoleic, palmitic and oleic acids together with small amounts of stearic, palmitoleic, myristic and lauric acids. This does not vary with the age of the culture. On the other hand, if fatty acid synthetase is extracted and assayed in vitro, an unusual product pattern is found, with lauric acid as the main compound (88%) and the rest is mainly decanoic acid. The assay contained acetyl-CoA and malonyl-CoA in a ratio of 1 : 5 i.e., as expected for lauric acid. The products from, for instance, the yeast enzyme can be varied in length by using different proportions of acetyl-CoA and malonyl-CoA. Large changes in the proportion do however result in a relatively slight change in product pattern; the same fatty acids are found, but in different yields (81).

Lauric acid is widely found in living organisms, but it always occurs as a minor constituent together with the common C_{16} and C_{18} acids. It is also found as a product from fatty acid synthetase, usually in quantities around 1% of the total fatty acids. This

fatty acid synthetase does thus have a product specificity, which is clearly different from the yeast enzyme. This may be an artifact of the environment in the in vitro assay, but it would also suit the biosynthesis of the alkyl citric acids since the two synthases do not accept acids longer than C₁₂ (IV, VII).

The formation of the C₁₆ and C₁₈ fatty acids in the mycelium remains to be explained. It can either be caused by a different fatty acid synthetase, which is less easily extracted, or the same multi-enzyme may give a different product spectrum in a different cellular environment. It is interesting to note the discrepancy between in vivo and in vitro chain length in the mammary gland enzyme in this context (82).

Relatively little is known about the properties of the fatty acid synthetase in this species, but it can be purified by ammonium sulphate precipitation, and it sediments within 4 h at 100 000 g, indicating a high molecular weight. There is no evidence of the requirement of an acyl carrier protein (83).

d. Decylcitrate synthase.

2-Decylcitrate oxaloacetate-lyase
(CoA lauroylating) EC 4.1.3.

Decylcitrate synthase (Fig. 4) is a new enzyme, which occurs only in the strain of *P. spiculisporum* that produces decylcitric acid.

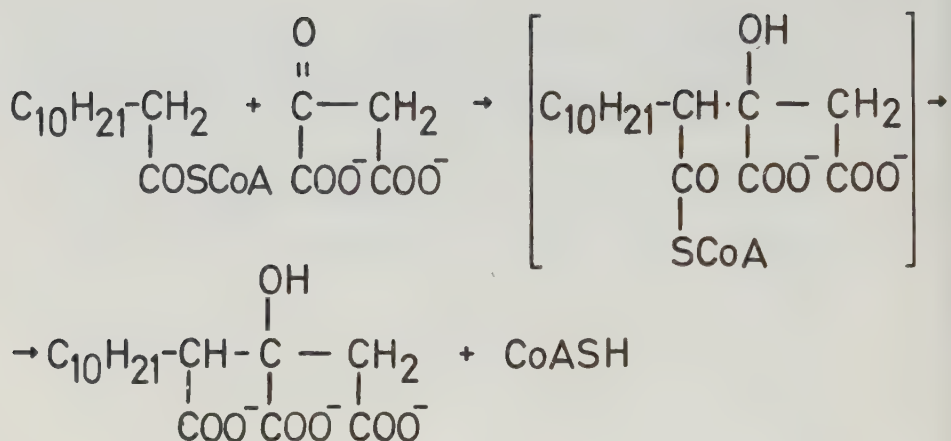


Fig. 4. Reaction of decylcitrate synthase.

No other enzymes of this type have been isolated, but the reaction seems to be analogous to that of citrate synthase. The enzyme requires the whole CoA molecule for activity, has the same narrow keto acid specificity as citrate synthase and does also seem to be a dimer (IV).

Special problems arise in the kinetics of decylcitrate synthase due to the substrate lauroyl-CoA. Fatty acyl-CoA are strongly polar substances forming very large micelles in water solution, and in dilute solution they cling to the water-air interface. Palmitoyl-CoA has been well studied in these respects by Cleland et al. (84,85) and he has determined the critical micelle concentration for various fatty acyl-CoA. Palmitoyl-CoA has been found to inhibit a large number of enzymes, and this has been assumed to be another consequence of its surface activity (86). The surface activity of lauroyl-CoA, however, is less pronounced, but it does exert special polar and surface

effects in enzymatic reactions, and the usual type of concentration dependence can not be expected. Evaluation of kinetic data has to be done with care both at high and low concentrations of the substrate. Since few enzymes utilising fatty acyl-CoA have been studied in solution - most of them are difficult to extract from particles such as microsomes (87) - the investigation of the lauroyl-CoA interactions with decylcitrate synthase is of interest.

The data have been interpreted in the following way: The apparent K_m for lauroyl-CoA is lower than $0,1 \mu M$ i.e., below the limit of the available techniques. This seems reasonable, since the enzyme probably has to compete with other proteins, which adsorb lauroyl-CoA, giving a very low concentration of free lauroyl-CoA in a concentrated cellular protein solution. At higher substrate concentrations the enzyme is inhibited. Using various techniques, the interactions have been summarised as follows:

- 1.) formation of a very strong binary complex,
- 2.) at higher concentrations of lauroyl-CoA, the tertiary structure of the enzyme is changed due to occupation of binding sites other than the active, but the activity is retained,
- 3.) at even higher concentrations the enzyme is totally denaturated.

Decylcitrate synthase is remarkably sensitive to the chain length of the acyl-CoA substrate. Only C_{12} and C_{10} are accepted in accordance with the product pattern of the fatty acid synthetase. ω -substituted acyl-CoA have also been synthesised, and the use of these analogues indicate that a hydrophobic carbon chain is essential for the substrate binding.

As with citrate synthase (88,32), decylcitrate synthase does not accept oxalacetate in the enol form (unpublished). The SH reagent sensitivity of the enzyme completely disappears when low amounts of lauroyl-CoA are added, and it can conveniently be assayed with the use of SH reagents. A similar substrate dependent SH sensitivity has been observed in mango fruit citrate synthase (69). The binary lauroyl-CoA complex may be a dead-end complex, as discussed in the next section, and it is also interesting that the kinetic data on pig heart citrate synthase can be interpreted as due either to a random ternary complex mechanism or to a compulsory-order mechanism, where oxalacetate binds first (89). Substrate inhibition with acyl-CoA is observed in that enzyme as well.

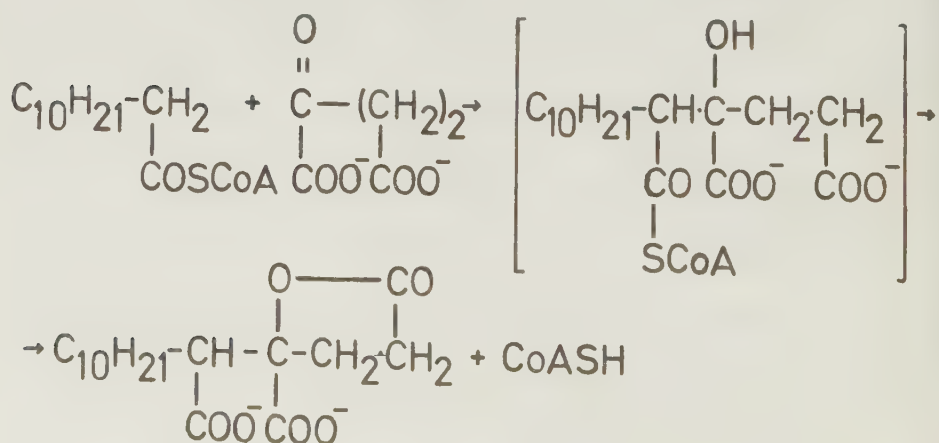


Fig. 5. Reaction of decylhomocitrate synthase.

e. Decylhomocitrate synthase.

2-Decylhomocitrate 2-oxoglutarate-lyase (CoA lauroylating). EC 4.1.3.

Decylhomocitrate synthase (Fig. 5) is very similar to decylcitrate synthase, as far as molecular size and fatty acyl-CoA specificity are concerned. Differences are noticeable of course in the keto acid requirements, where this enzyme accepts only ketoglutarate. Oxalacetate is a strong competitive inhibitor in decylhomocitrate synthase, whereas the reverse does not hold in decylcitrate synthase. The SH reagent sensitivity of decylhomocitrate synthase is in principle the same as in decylcitrate synthase i.e., lauroyl-CoA protects the enzyme against inactivation, but to a much smaller extent. In the presence of the competitive inhibitor oxalacetate as well, the enzyme is considerably better protected. This has been taken as evidence that the enzyme may work by a compulsory-order ternary complex mechanism, where ketoglutarate binds first to the enzyme (Fig. 6). The dissociation constant of the enzyme is too high, as judged from the SH protection, to account for the observed apparent K_m for lauroyl-CoA, which is lower than $1 \mu\text{M}$, and this excludes that a mechanism where lauroyl-CoA binds first, takes a major part of the reaction. In decylcitrate synthase a similar mechanism may well be operating. This enzyme has a much lower dissociation constant for the binary lauroyl-CoA complex, and the assumption of this as a dead-end complex would explain the strong substrate inhibition and other phenomena observed in decylcitrate synthase. A close parallel to the differences in binding of acyl-CoA in presence and absence of keto

acid has recently been reported by Weidman and Drysdale from pig heart citrate synthase (90).

It is interesting to find two enzymes so similar in strains of the same species, and yet with absolute specificity for slightly different keto acids. It has been suspected that decylcitrate synthase may have arisen through a minor mutation in the active site of decylhomocitrate synthase, which is not disproved by the similarities in size, acyl-CoA specificity and other properties. An immunological comparison has unfortunately failed, since the enzymes are poor antigens, but inspection of the tryptic peptides reveals large differences in the peptide pattern, indicating different amino acid composition in more than one position. As the enzymes have not been purified to the highest degree, and peptide analysis is an unspecific method, a definite conclusion can not be made at this point.

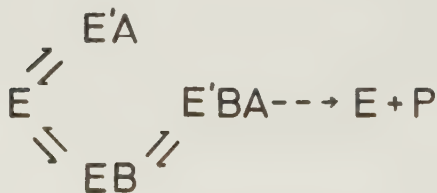


Fig.6. Proposed mechanism of the *P. spiculisporum* synthases.

A=acyl-CoA, B= keto acid.

7. REMARKS ON THE ENZYME ISOLATIONS

Since relatively few enzymes have been isolated from moulds except *Neurospora*, a few comments on the procedures used in *Penicillium spiculisporum* may be of interest. For good recovery, it is essential to wash the mycelium free of constituents in the medium. In this species, the detergents themselves are efficient protein denaturants, but there seem to be other inhibitors as well. In the isolation of decylcitrate synthase often no activity is found in the crude extract; however, activity appears when the enzyme has been purified by ammonium sulphate precipitation. Both strains grow as a rather uniform dispersion of cells or rather small pellets, which simplifies the thorough washing of the mycelium. Mycelium, which has been washed with distilled water and buffer, can generally be stored frozen for months, although with ATP citrate lyase the activity decreases noticeably.

Among methods of homogenisation, sand grinding, blending in a Waring blender or Omnimixer and processing through an X-press or Ribi press have been used, and the best results have been encountered with the Ribi Cell Fractionator. It has advantages both in so far as efficiency and convenience on different scales are concerned. The use of a high ionic strength in the homogenisation buffer has been found to improve the extraction of the enzymes from the cells.

One characteristic, common to most of the enzymes investigated, is a considerable tendency towards aggregation, which is promoted by low ionic strength

and low protein concentration. This has resulted in abandonment of the use of extensive dialysis against dilute buffers and ion exchange columns with large volumes of eluting buffer. Sensitivity towards aerial oxidation of SH groups has also been found with almost all of the enzymes. The aggregation and SH group oxidation have generally led to poor yield in steps involving column chromatography, and have not allowed the enzymes to be purified to absolute homogeneity. Ammonium sulphate precipitation has given excellent recoveries, and gel filtration and hydroxylapatite chromatography with fast flow rates and steep gradients have been useful, whereas adsorption on DEAE-cellulose or DEAE-Sephadex has never been reversible by increasing the ionic strength. Density gradient centrifugation and ultrafiltration have been found to be mild, purification methods resulting in good recoveries of the enzyme with high activities.

No satisfactory storage conditions for the enzymes have been found. Decylcitrate synthase, after two years storage at -18°C , actually retains 25% of its activity, however it then consists of a whole range of molecular sizes up to M_w 500 000 or more. For measurements where the native configuration has been necessary, the preparations have been used for only a few days up to a week and then stored at 0°C at high ionic strength and with high protein concentration.

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